Observations on the Rapid Size-Exclusion Chromatography of Proteins

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Summary

The retention behaviour of reference proteins on commercial siliceous size-exclusion supports was studied. Sorption was observed on both surface modified and unmodified supports. When sodium dodecylsulfate was added to the aqueous mobile phase, normal elution patterns were found. With this system, proteins, such as those isolated from different alfalfa genotypes, may be compared rapidly. Comparisons were facilitated by use of on-line central data processing capability.

Introduction

Modern high-performance liquid chromatography (HPLC) has tremendous potential for separating proteins rapidly, although methods for its routine use are emerging only slowly. Size-exclusion chromatography (SEC) is of great interest to biochemical investigators because molecular weight information may be derived from it and because, ideally, all solutes are eluted conveniently in a single column volume. Small particle siliceous-supports have been utilized for rapid size-exclusion in aqueous solvents. Considering that the surface areas of such materials are in the range of 100–300 m^2/g with a surface silanol concentration of about 4–6 functional groups per 100 $Å^2$ and that the areas of protein molecules are about a thousand $Å^2$, sorption at the surfaces would be expected. Since such a large number of interaction sites exists, the binding of proteins is suggested even when organic moieties are covalently bound to the support surface to prevent sorption. In this report, the properties of several commercial supports are examined and their potential for the examination of leaf-proteins isolates is explored.

Experimental

Chromatographic System

Columns were assembled from 316 stainless steel tubing (0.64 cm OD, 0.41 cm ID) that had been cut to the desired length and thoroughly washed, flushed with acetone, then hexane, and dried. Fritted disks (0.25 μm pore diameter) were pressed into holes countersunk into the outlet end of the tubing. Stainless steel fittings and tubing were used throughout the system. Connections between columns in series and to the detector were of capillary tubing (0.16 cm OD, 0.02 cm ID) to minimize dead volume. A Milton-Roy* minipump provided a constant flowing mobile phase. A pressure-limiting switch and gauge (Barksdale Inc.), placed in the line for safety reasons, also provided sufficient pulse dampening. The detector was a high-performance, fixed wavelength (254 nm) du Pont photometer. A fixed volume (200 mm^3) loop injector (Valco, Inc.) was employed.

Packings

Several commercial SEC packings were used in this study. System A consisted of a 1 m column packed with glyceryl-propylsilyl controlled-pore glass (GPS/CPG) [1] provided by Pierce Chemical Company, 37–75 μm particle diameter, 250 Å pores coupled with a similar column packed with 550 Å pore particles. System B was as above but consisted of a series of four columns. Two were packed with 250 Å pore particles, one with 550 Å pore particles and one with 1500 Å pore particles. System C was a commercially packed microparticulate (~8 μm) silica column (SEC-500, du Pont Inc.).

Mobile Phase

Phosphate buffers (0.005 mol dm^{-3}, pH = 7.5) containing 0.02 % (w/v) NaN_3 and 0, 0.1, 0.5 molar in sodium sulfate were used to study sorption effects on system A. Subsequent chromatograms were developed with the same phosphate buffer without the salt but with 1 % (w/v) sodium dodecylsulfate (SDS).

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Protein Sources

Reference proteins were obtained from either Pharmacia Chemicals or Polysciences Inc. (USA). The alfalfa protein isolate (API) was prepared as follows. Alfalfa plants were grown by Dr. James Elgin (Beltsville Agricultural Research Center, USDA, Beltsville, Md.) and the leaves were quick-frozen in liquid nitrogen at harvest. To prepare the extract, the leaves were triturated with cold 0.05 mol dm^{-3} tris-buffer (pH 9). The extracts were clarified by filtration through cheesecloth and ultracentrifugation (10 min at 20 K g, 0 °C). The clear extract was then passed through a 2.5 x 30 cm column packed with Sephadex G-50-coarse (Pharmacia) to separate the proteins from phenols and other smaller molecules which degrade the proteins. The protein fraction eluted near the void volume. It was lyophilized and stored at −20 °C until used. The alfalfa protein isolates were further fractionated according to the scheme shown in Fig. 1.

In all cases proteins were dissolved in the appropriate mobile phase to a concentration of 1 mg/cm^3 and filtered through a 0.45 μm bacteriological filter prior to injection into the chromatograph. 200 mm^3 were injected.

Computer Analysis

The analog signal from the fixed wavelength detector was amplified 500 times and transmitted through a shielded cable to a Modcomp III (sampling rate 5 points/s) data acquisition unit. Computations were carried out batchwise on an IBM 1130 computer. Void volume (in time units) and total volume were entered off-line, and the chromatograms were replotted in terms of the distribution coefficient K_{AV}. Blue-dextran 2000 and sodium azide were used to determine void (V_O) and total (V_T) volumes, respectively. Moment analyses in terms of K_{AV}, as suggested by Fishman [2] were performed, and area distributions were calculated.

$(NH_4)_2SO_4$ Fractionation

50 mg of stored, dried soluble protein (API) is redissolved in 15 cm^3 0.05 mol dm^{-3} tris buffer, centrifuged and supernatant is removed

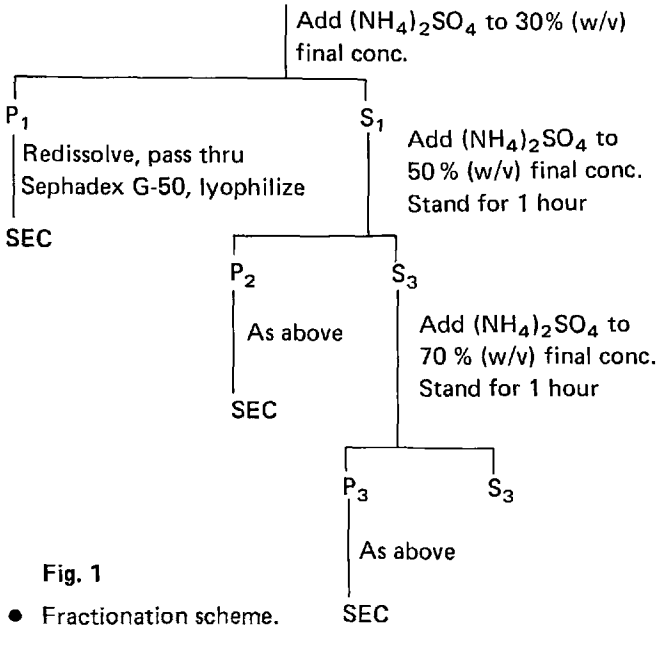

Fig. 1

- Fractionation scheme.

Results and Discussion

The elution behaviour of several proteins from a commercial surface modified support (system A) is plotted in Fig. 2. At low ionic strength chymotrypsinogen was not eluted. At higher ionic strengths, it was eluted, but the elution volume was never less than the total column volume. This demonstrates the affinity of this particular support for some proteins. Under the conditions described, these proteins were never observed to elute in inverse order of molecular weight as one might expect in size exclusion chromatography, and at the higher ionic strengths they eluted in reverse order of the expected. The increase in retention time of ovalbumin and ribonuclease with increasing salt concentration (increasing surface tension) suggests that hydrophobic interactions may be taking place [3], although the order is not the same as that found by Hofstee for the nonionic sorption of proteins on alkyl-substituted agarose [4]. Addition of 1% (v/v) ethylene glycol to the mobile phase did not produce regular size-exclusion chromatography (SEC) elution patterns. When a phosphate-sodium dodecylsulfate (SDS) buffer is used with the same siliceous supports, the expected elution order is observed (Fig. 3). Aldolase elutes under these "denaturing" conditions at the volume corresponding to the molecular weight of the monomer (40 K), although it exists usually as a tetramer in other buffers [2]. Previous results [5,6] also have shown that the expected order is obtained with such denaturing mobile phases. This order also was observed when phosphate-SDS mobile phase was used with an unmodified siliceous support (system C).

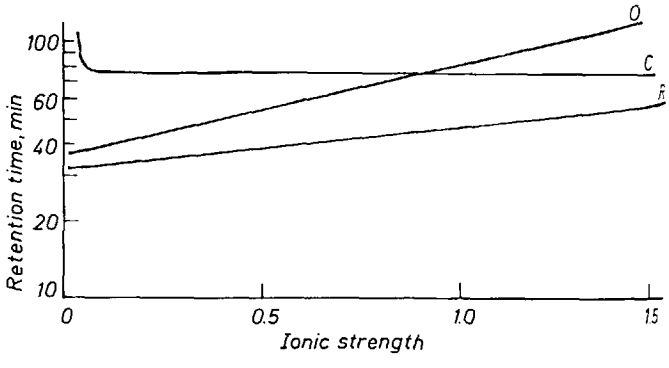

Fig. 2

- Elution of protein from surface modified controlled pore glass (system A). (O) ovalbumin (MW 45 K Daltons); (C) chymotrypsinogen (25 K); (R) ribonuclease (13.7 K). Time to elute total volume marker (NaN_3) was 53 min. Mobile phase: 0.005 mol dm^{-3} phosphate buffer (pH 7.5) + Na_2SO_4 to adjust ionic strength.

While it would be advantageous to carry out the chromatography of protein isolates under nondenaturing conditions so that fractions could be recovered to assess enzymatic activity, useful analytical information can be obtained under denaturing conditions.

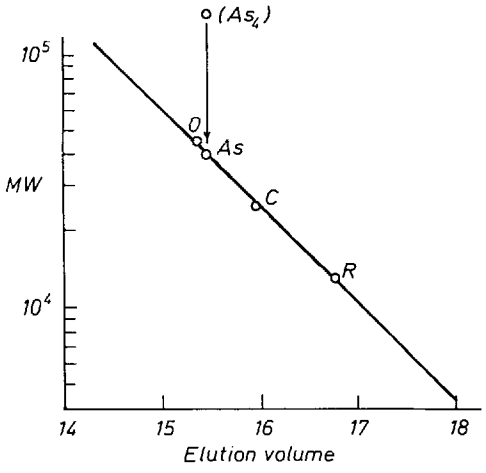

Fig. 3

• Elution of protein from surface modified controlled pore glass (system B) with SDS-phosphate buffer. 0.005 mol dm^{-3} phosphate (pH 7.5) + 1% SDS. (As) aldolase (40 K Daltons); (As_4) tetramer of aldolase (not observed in this system). The other symbols as in Fig. 2.

An example is given in Fig. 4 which shows chromatograms obtained on both a surface modified, controlled pore-glass column and a microparticulate silica column of the same sample, a soluble protein fraction isolated from alfalfa leaves. Each chromatogram took about 0.5 hour to complete. Visual examination of chromatograms, as well as comparison of moments and area distribution of the complex profiles, indicated little difference between several genotypes studied. When the isolates were fractionated by use of the ammonium sulfate precipitation scheme, more pronounced differences were observed (Fig. 5) between corresponding fractions of different genotypes. The areas of the actual chromatograms were normalized and replotted for this figure in terms of the distribution coefficient K_{AV} by use of the on-line data acquisition system. Examination of the area distribution showed that the P-1 fraction from the one genotype had more protein in the 0.4–0.5 K_{AV} region than the corresponding fraction of the other genotype, while the latter had more protein in the 0.7 region. This was reflected in a first moment apparent molecular weight of 25 K Daltons for the former fraction and 17 K Daltons for the latter. The biochemical significance of these differences requires further study.

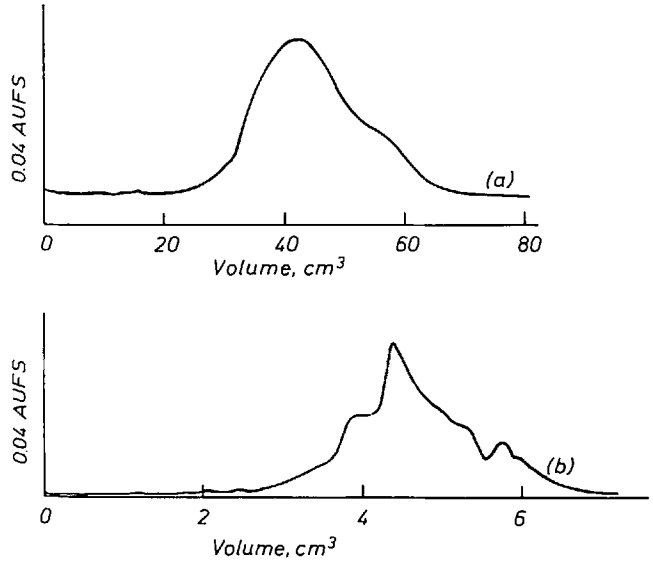

Fig. 4

• SEC of alfalfa protein isolate. Mobile phase: same as Fig. 3. (a) system B: V_O = 32.4 cm^3; V_T = 56.0 cm^3; (b) microparticulate silica (system C): V_O = 3.12 cm^3; V_T = 5.69 cm^3.

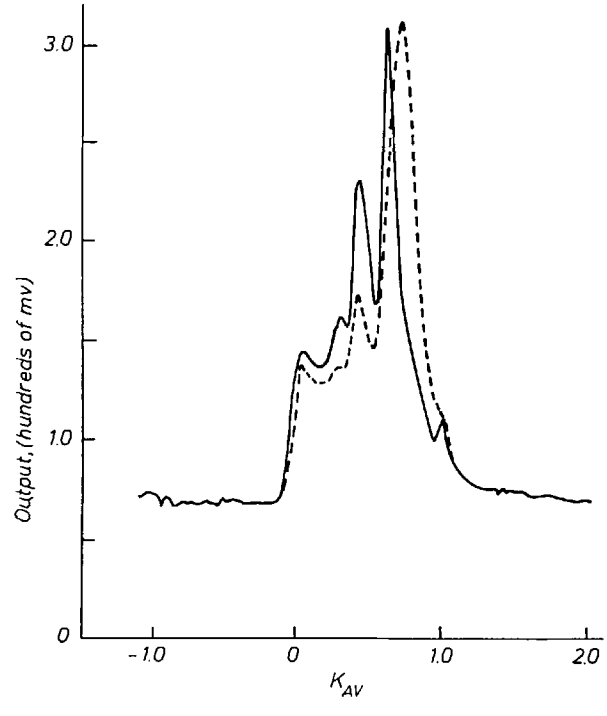

Fig. 5

• Comparison of P_1 fractions from ammonium sulfate fractionation of protein isolates from two different genotypes. Conditions as in Fig. 4 (b). Chromatogram replotted in terms of K_{AV} with on-line central data system.

Some care is needed, however, in interpreting size-exclusion chromatograms of proteins obtained with siliceous supports. Sorption occurred on both the surface-modified and unmodified supports which we studied. Of the mobile phase modifiers evaluated, only SDS produced the expected elution order. Addition of salt did not eliminate sorption effects so that molecular weight information obtained would have no value. The lack of long-range stability of the columns was encountered in these studies. The need for frequent replacement may diminish their utility for some routine applications.

In spite of the limitations indicated, the utility of modern size-exclusion chromatography for the investigation of complex protein isolates is demonstrated in this report.

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References

[1] *F. E. Regnier* and *R. Noel*, J. Chromatogr. Sci. **14**, 316 (1976).

[2] *M. L. Fishman*, Anal. Biochem. **74**, 41 (1976).

[3] *C. Horvath, W. Melander* and *I. Molnar*, J. Chromatogr. **125**, 129 (1976).

[4] *B. H. J. Hofstee* and *N. F. Otillio*, J. Chromatogr. **159**, 57 (1978).

[5] *R. A. Barford, J. Kupec* and *M. L. Fishman*, J. Water Poll. Control Fed. **49**, 764 (1977).

[6] *R. J. Blasgrove* and *M. J. Frankel*, J. Chromatogr. **132**, 399 (1977).

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